

AD_____

Award Number: DAMD17-00-1-0503

TITLE: Mechanisms Underlying the Increased Susceptibility of the
Immature Mammary Gland to Selected Carcinogens

PRINCIPAL INVESTIGATOR: Jennifer L. Ariazi
Doctor Michael Gould

CONTRACTING ORGANIZATION: University of Wisconsin System
Madison, Wisconsin 53706-1490

REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20011127 103

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2001	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jun 00 - 31 May 01)		
4. TITLE AND SUBTITLE Mechanisms Underlying the Increased Susceptibility of the Immature Mammary Gland to Selected Carcinogens		5. FUNDING NUMBERS DAMD17-00-1-0503		
6. AUTHOR(S) Jennifer L. Ariazi Doctor Michael Gould				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Wisconsin System Madison, Wisconsin 53706-1490 E-Mail: jariazi@students.wisc.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The immature human breast is more susceptible than the mature breast to the carcinogenic effects of ionizing radiation. Studies have demonstrated that immature rat mammary epithelial cells (RMECs) are more susceptible than their mature counterparts to the cytolethal effects of ionizing radiation and N-nitroso-N-methylurea (NMU) but not dimethylbenzanthracene (DMBA). The work reported here was undertaken to further explore this age-differential carcinogen-specific mammary epithelial cell susceptibility. The goal is to determine whether immature RMECs are also more susceptible than mature RMECs to the mutagenic effects of NMU, possibly due to differences in the repair of NMU-induced DNA damage. Using the Big Blue [®] transgenic mutagenesis assay system, it was discovered that more mutants persist in immature than mature RMECs following NMU treatment <i>in vivo</i> . Comet assays revealed that while there were no age-related differences immediately following NMU treatment <i>in vitro</i> , immature but not mature RMECs display increased tail moments beginning two hours following NMU treatment. Apoptosis does not cause the difference. Mature RMECs mimicked the immature RMEC response after benzylguanine pretreatment, which inhibits methylguanine methyltransferase (MGMT); such treatment did not affect immature RMECs. The data support the working hypothesis that immature RMECs are deficient in MGMT activity relative to mature RMECs.				
14. SUBJECT TERMS NMU, Big Blue rat, comet assay, mammary gland, mutagenesis, DNA repair, risk as a function of age, breast cancer prevention			15. NUMBER OF PAGES 10	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....5

Reportable Outcomes.....5

Conclusions.....5

References.....6

Appendices.....7

INTRODUCTION

It is known that the immature human breast is more susceptible than the mature breast to the carcinogenic effects of ionizing radiation [1, 2]. Rat studies have demonstrated that immature rat mammary epithelial cells (RMECs) are more susceptible than their mature counterparts to the cytolethal effects of ionizing radiation, and preliminary data indicate that this phenomenon extends to N-nitroso-N-methylurea (NMU) but not dimethylbenzanthracene (DMBA) [3, 4]. The work reported here was undertaken to further explore this age-differential carcinogen-specific mammary epithelial cell susceptibility. Specifically, the goal is to determine whether immature RMECs are also more susceptible than mature RMECs to the mutagenic effects of NMU, possibly as a result of age-related differences in the ability to repair NMU-induced DNA damage. Persistent mutational differences are addressed using the Big Blue transgenic mutagenesis assay system to examine the mutant frequencies of RMECs from immature and mature rats treated with NMU *in vivo*. Mutation frequencies and spectra will be compared once the mutants are sequenced. Age-related differences in the level of early potentially mutagenic DNA damage are addressed using comet assays, and variations in their repair are addressed using both comet and apoptosis assays. Finally, age-related differences in the ability to repair NMU-induced DNA damage will be assessed by directly assaying methylguanine methyltransferase (MGMT), the enzyme primarily responsible for the repair of such potentially mutagenic alkylation damage.

BODY

Demonstrate that NMU treatment *in vitro* recapitulates *in vivo* survival phenomenon

No progress has been made in demonstrating that NMU treatment *in vitro* recapitulates the *in vivo* survival phenomenon.

Long-term persisting mutations in RMECs of immature and mature rats

Considerable progress has been made in the study of persisting mutations in the RMECs of immature and mature animals. Three sets of immature and mature Big Blue rats (3 rats per group) have been treated with NMU or vehicle control and sacrificed after 1-, 3-, or 5-week expression periods. Mammary epithelial cells, as well as livers and spleens, have been collected from all these rats and are stored at -80°C . RMEC mutant frequencies from one full set of rats (immature and mature, control and NMU-treated) have been determined and the results are presented (Figure 1; all figures are in the appendix). Approximately 300,000 plaques have been scored for each condition (Figure 2). Exceptions are mature control RMECs. Although multiple packaging reactions were performed to the point of exhausting the DNA from these samples, a maximum of 150,000 plaques was achieved with these samples. This difficulty in packaging mammary epithelial cell DNA has been reported by another group, but the reason for the difficulty of packaging mammary epithelial cell DNA is unknown [5]. NMU induced mutants in immature and mature RMECs at all expression periods. Statistically significant differences (statistical analysis performed by biostatistician Mary Lindstrom) were found between immature and mature NMU-treated RMECs at all time points examined. Additionally, there is no statistically significant difference between the 3- and 5-week expression period mutant frequencies, indicating that the mutant frequencies have plateaued. Verified mutant plaques were cored and are stored awaiting sequence analysis.

Short-term mutations and their repair in RMECs from immature and mature rats

Significant progress has been made in the study of early potentially mutagenic DNA damage and its repair in RMECs from immature and mature cells. Comet assays were performed using *in vitro* NMU-treated immature and mature RMECs (Figure 3). NMU treatment induces increased tail moments relative to controls in both immature and mature RMECs. Initial (time 0 and 1 hour post-NMU treatment) measurements indicate no difference between immature and mature RMECs. However, beginning two hours following NMU treatment, the immature RMECs display a significant increase in NMU-induced tail moments relative to mature RMECs. Apoptosis is not responsible for the age-related difference, as no differences in apoptosis were found prior to 17 hours following NMU treatment (Figure 4).

Mechanism of removal of O⁶-methylguanine, a known cytotoxic lesion

Promising initial results have been revealed in the study of the mechanism of removal of O⁶-methylguanine, a known cytotoxic lesion. Comet assays have been performed on RMECs pretreated with benzylguanine to inhibit MGMT *in vitro* (Figure 5). Such treatment does not affect the tail moments of immature RMECs. However, MGMT inhibition by benzylguanine pretreatment in mature RMECs causes them to recapitulate the immature RMEC tail moment response. Immature and mature RMECs are being collected and frozen for future MGMT activity analyses.

KEY RESEARCH ACCOMPLISHMENTS

- Immature RMECs have higher mutant frequencies than mature RMECs at all timepoints analyzed.
- Mutant frequencies have plateaued.
- There is no initial (0 and 1 hour timepoints) difference between immature and mature RMEC tail moments after NMU treatment.
- Immature but not mature RMEC tail moments increase two hours following NMU exposure *in vitro*.
- Mature RMECs exhibit the tail moment increase beginning two hours after NMU treatment *in vitro* if their methylguanine methyltransferase has been inhibited by benzylguanine pretreatment.
- Methylguanine methyltransferase inhibition by benzylguanine pretreatment minimally affects immature RMEC tail moments while causing the mature RMECs to recapitulate the immature response.

REPORTABLE OUTCOMES

Work supported by this fellowship was presented at the 40th annual meeting of the Society of Toxicology, March 25-29, 2001, in San Francisco, CA. It was a platform presentation in the Carcinogenesis category. Also from that meeting, the abstract won the first prize in the Carcinogenesis Special Section Student awards. The title was "Increased Susceptibility of Immature Rat Mammary Epithelial Cells to the Cytotoxic, Carcinogenic, and Mutagenic Effects of N-nitroso-N-methylurea."

CONCLUSIONS

Results obtained to date support the hypothesis that immature RMECs are more susceptible than their mature counterparts to the mutagenic effects of NMU. Following *in vivo* NMU exposure, immature RMECs exhibit higher mutant frequencies than mature RMECs at all timepoints examined, and the mutant frequencies have plateaued (Figure 1). This indicates that RMECs treated with an alkylating agent when immature harbor greater levels of persisting mutations than RMECs treated when mature. This is extremely relevant in light of the known elevated susceptibility of the immature human breast to carcinogenic insult by ionizing radiation.

Based on the results of the comet assays, these persistent differences are not likely due to differences in initial DNA damage, since there were no tail moment differences between immature and mature RMECs at time 0 or 1 hour following NMU treatment (Figure 3). Beginning two hours after NMU treatment *in vitro*, the immature RMECs exhibit increased tail moments, while the mature tail moments remain steady. Apoptosis does not account for these differences (Figure 4). Because inhibition of MGMT by benzylguanine causes the mature RMECs to exhibit increased tail moments similar to those produced by immature RMECs, the age-differential response appears related to MGMT (Figure 5). Taken together, these data suggest that the immature RMEC is deficient in methylguanine methyltransferase activity relative to the mature RMEC. If O⁶-methylguanine is still present in the DNA when it is replicated, a GC → AT transition mutation can occur, as shown in Figure 6. Both base excision repair and mismatch repair recognize and act on the G-T basepair. Consistent with the tail moment increase seen beginning two hours after NMU treatment of immature RMECs, both repair pathways introduce DNA strand breaks in order to correct the mutation caused by O⁶-methylguanine. Both, too, are lower fidelity repair mechanisms than methylguanine methyltransferase's direct reversal [6]. These repair processes are thought to contribute to alkylation-induced cytotoxicity by introducing DNA strand

breaks, particularly when they participate in futile cycling [7]. The data reported here support the working hypothesis that O⁶-methylguanine in immature RMECs is not efficiently repaired by MGMT as it is in mature RMECs. While base excision or mismatch repair subsequently act on the O⁶-methylguanine-thymine basepair, more mutations remain unrepaired in immature than mature RMECs.

So what? New insight into the developmental regulation of an important DNA repair pathway, methylguanine methyltransferase, is important for basic biology. Moreover, in light of the known age-differential susceptibility of the human breast to the carcinogenic effects of ionizing radiation (which does produce lesions repaired by methylguanine methyltransferase) these results could be the impetus for a new line of research into human breast cancer susceptibility. Humans are, after all, exposed to alkylating agents in the diet and cigarette smoke [8-10]. Once completed, the results of these studies will support the value of studies into the expression and activity of methylguanine methyltransferase in the human immature and mature breast. Perhaps those studies will find activity differences analogous to those seen in the rat...and suggest a new target for chemoprevention studies.

REFERENCES

1. Tokunaga, M., et al., *Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950-1980*. Radiat Res, 1987. **112**(2): p. 243-72.
2. Hancock, S.L., M.A. Tucker, and R.T. Hoppe, *Breast cancer after treatment of Hodgkin's disease*. J Natl Cancer Inst, 1993. **85**(1): p. 25-31.
3. Haag, J.D. and M.N. Gould, *Cytotoxicity in Rat Mammary Epithelial Cells from Mature and Immature Rats Exposed to 80 mg/kg NMU or DMBA for 24 Hours*. Pers Comm, 1998.
4. Shimada, Y., et al., *Age and radiation sensitivity of rat mammary clonogenic cells [published erratum appears in Radiat Res 1994 Jul;139(1):128] [see comments]*. Radiat Res, 1994. **137**(1): p. 118-23.
5. Manjanatha, M.G., et al., *DNA adduct formation and molecular analysis of in vivo lacI mutations in the mammary tissue of Big Blue rats treated with 7, 12-dimethylbenz[a]anthracene*. Carcinogenesis, 2000. **21**(2): p. 265-73.
6. Singh, J., L. Su, and E.T. Snow, *Replication across O6-methylguanine by human DNA polymerase beta in vitro. Insights into the futile cytotoxic repair and mutagenesis of O6-methylguanine*. J Biol Chem, 1996. **271**(45): p. 28391-8.
7. Bignami, M., et al., *Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of methylating agents*. Mutat Res, 2000. **462**(2-3): p. 71-82.
8. Hecht, S.S., *DNA adduct formation from tobacco-specific N-nitrosamines*. Mutat Res, 1999. **424**(1-2): p. 127-42.
9. Georgiadis, P., et al., *Ubiquitous presence of O6-methylguanine in human peripheral and cord blood DNA*. Cancer Epidemiol Biomarkers Prev, 2000. **9**(3): p. 299-305.
10. Lijinsky, W., *N-Nitroso compounds in the diet*. Mutat Res, 1999. **443**(1-2): p. 129-38.

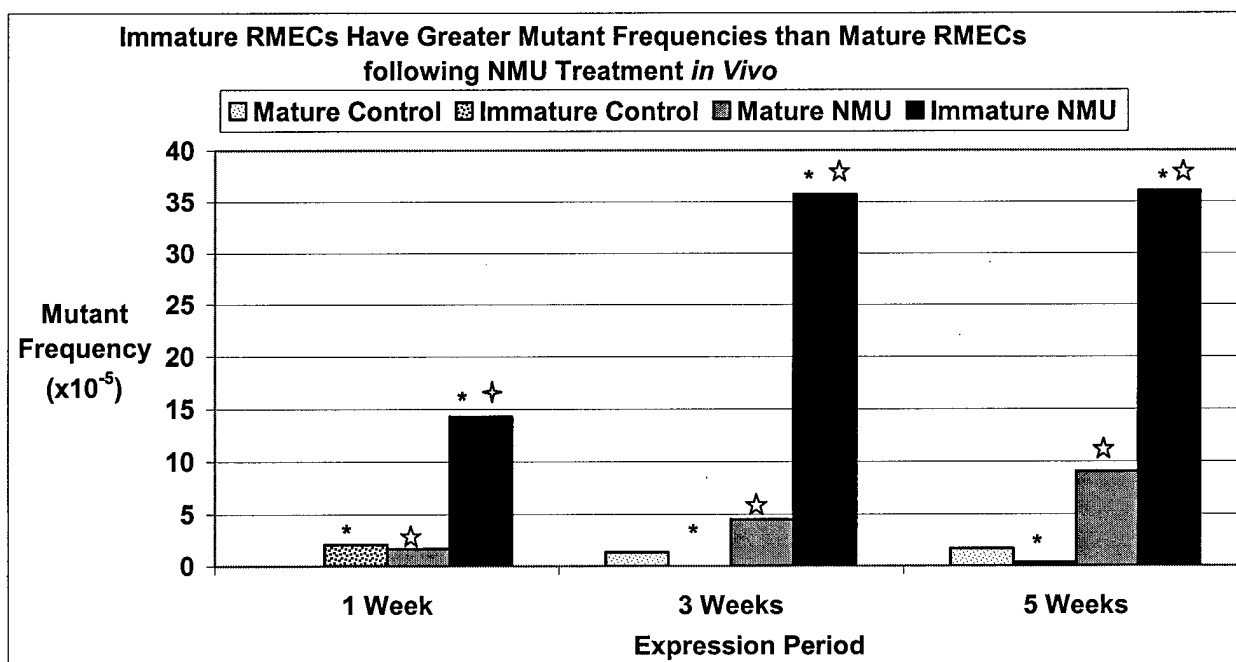


Figure 1. Immature (3 week old) and mature (8 week old) lacI (Big Blue[®]) transgenic rats were treated with 50 mg/kg N-nitroso-N-methylurea (NMU) by tail vein injection. After 1, 3, or 5 weeks, groups of three rats per age were sacrificed, and their mammary epithelial cells were isolated and pooled. This was necessary to isolate sufficient genomic DNA for packaging. Mutant frequencies were calculated using verified mutants only. Immature rat mammary epithelial cells (RMECs) have greater mutant frequencies than mature RMECs at all timepoints. Statistical significance was determined (by biostatistician Mary Lindstrom) using a generalized linear model for binomial data. *Different from mature, $p < 0.001$. ☆ Different from control, $p < 0.001$. † Different from control, $p = 0.01$.

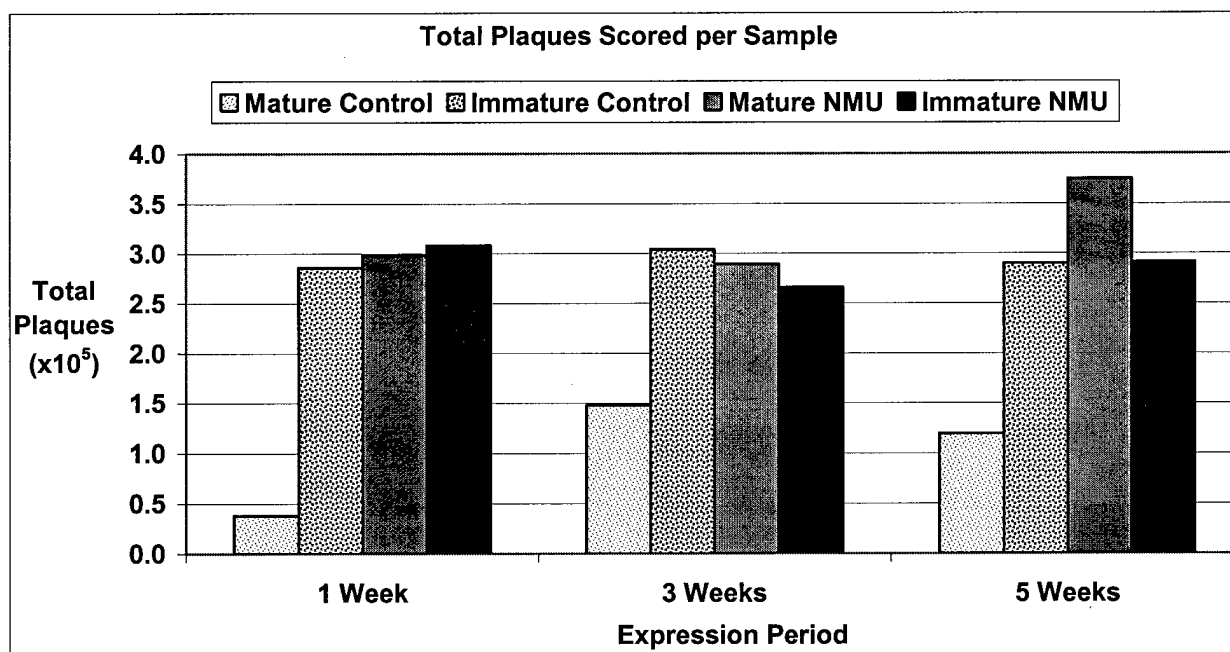


Figure 2. The total number of plaques plated is presented. At least 2.5×10^5 plaques were plated for all but the mature control samples. Most achieved $\sim 3.0 \times 10^5$ plaques.

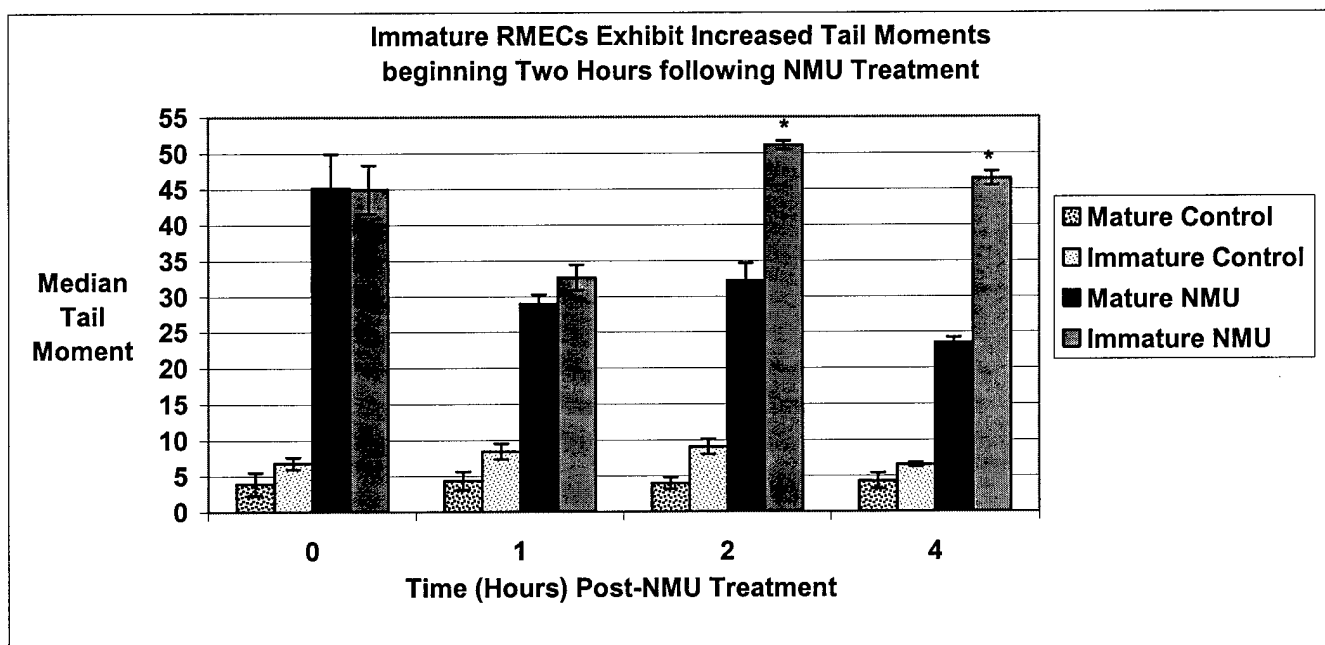


Figure 3. Mammary epithelial cells from immature (3 week old) and mature (8 week old) Fischer 344 rats were cultured overnight. They were treated with 100 μ M N-nitroso-N-methylurea (NMU) in medium or with medium alone for thirty minutes at 37°C. They were washed and provided fresh medium for the indicated times at 37°C. Single cell suspensions were made and alkaline comet assays were performed. Three slides per sample were prepared. Fifty comets per slide were scored, and median tail moments were determined. The means and standard deviations of the three values are presented. Statistical significance was determined using a paired, 1-tailed Student's t test. Tail moments of all NMU treated cells were statistically significantly different from tail moments of their respective control treated cells, $p < 0.005$. *Different from mature, $p < 0.005$.

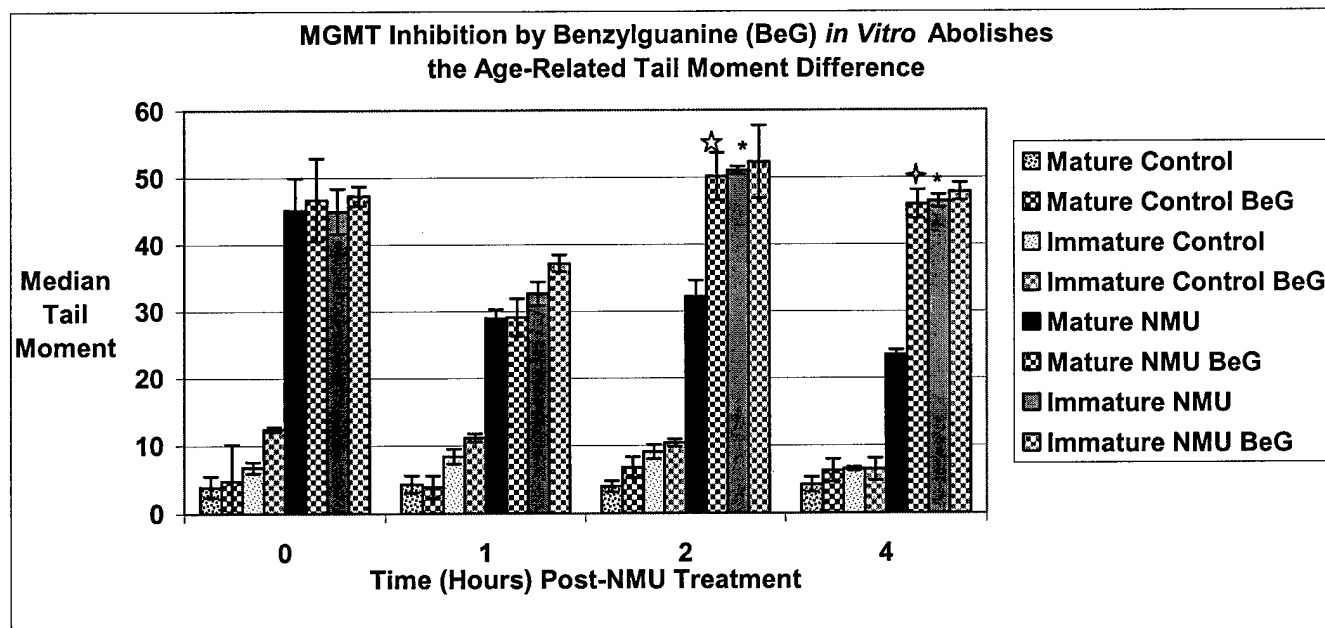


Figure 4. Cells were pretreated for two hours with 50 μ M BeG in medium or equivalent volume DMSO vehicle in medium. They were rinsed and treated with NMU as described above. Vehicle controls are those presented in A. DMSO treatment does not affect tail moments (data not shown). Statistical significance was determined using a paired, 1-tailed Student's t test. Tail moments of all NMU treated cells were statistically significantly different from tail moments of their respective control treated cells, $p < 0.005$. *Different from mature, $p < 0.005$. ☆Different from DMSO, $p < 0.03$. †Different from DMSO, $p < 0.002$.

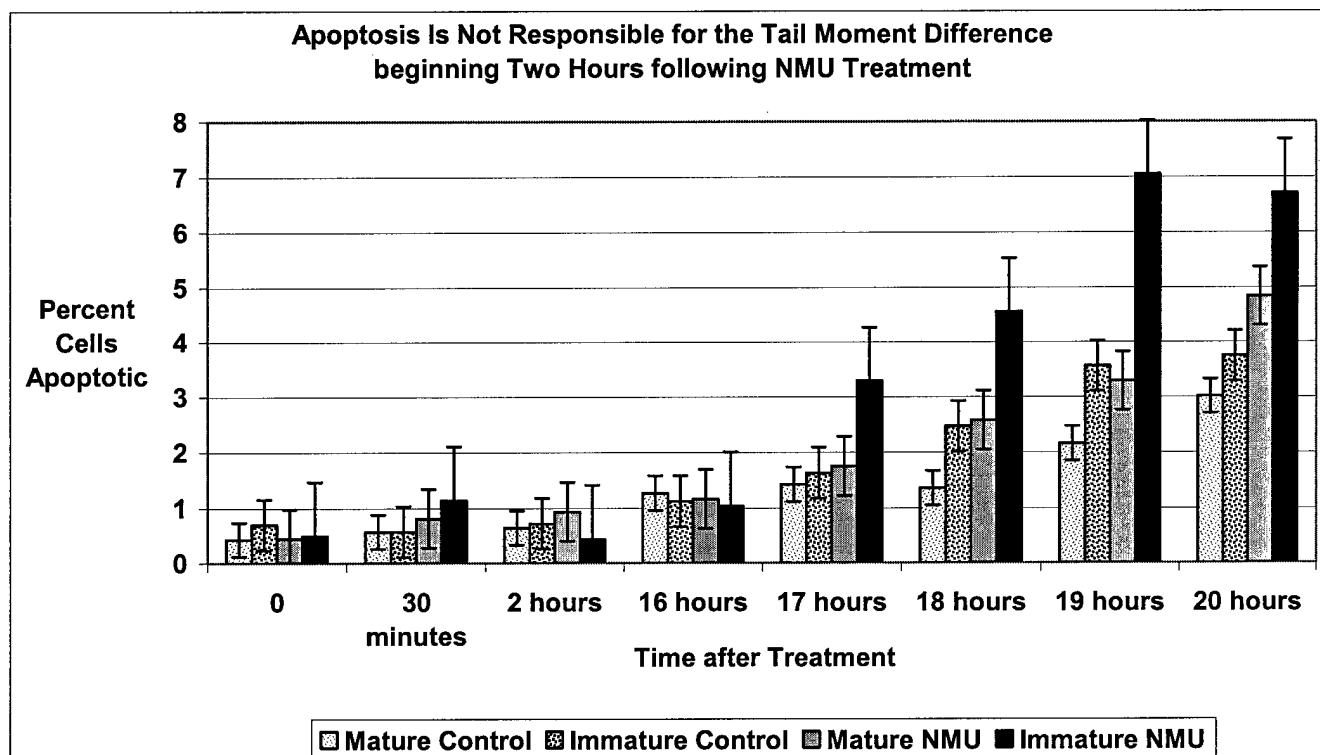


Figure 5. Mammary epithelial cells from immature (3 week old) and mature (8 week old) Fischer 344 rats were cultured overnight. The cells were treated with 100 μ M N-nitroso-N-methylurea (NMU) in medium or medium alone for 30 minutes at 37°C. Cells were washed and provided with fresh medium for the indicated times at 37°C. Cells were prepared as for a comet assay and stained with Hoescht stain. At least 400 cells were counted for each sample and the percentage of apoptotic cells \pm standard error of the mean are presented.

Mutagenic Potential of O⁶-Methylguanine

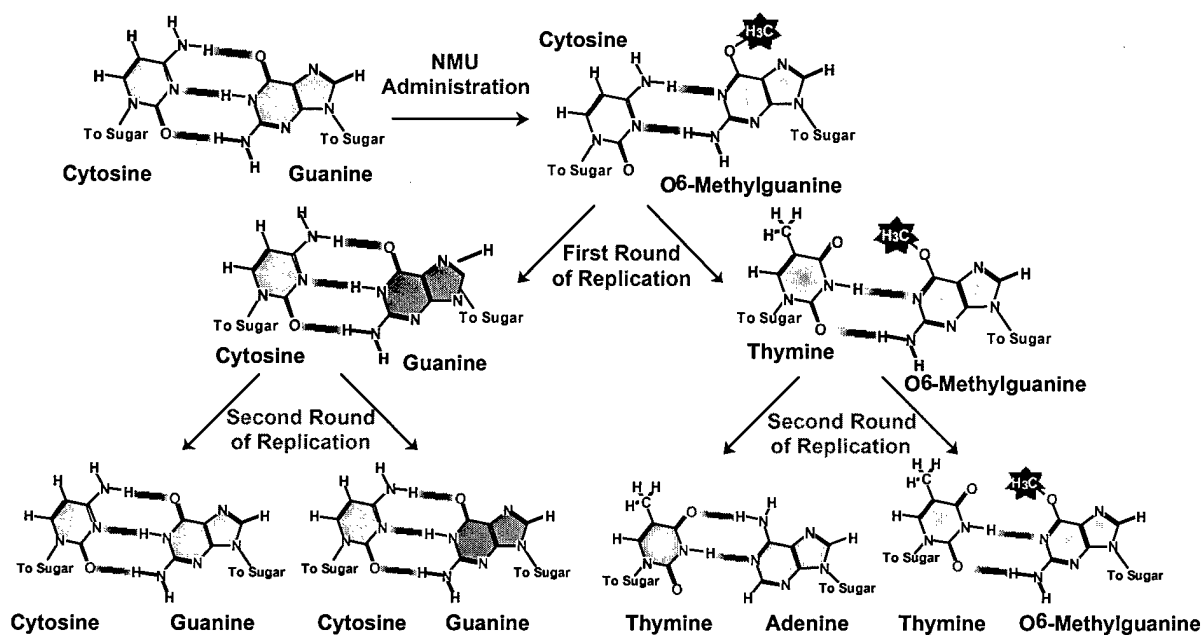


Figure 6. Administration of NMU results in the formation of O⁶-methylguanine. DNA polymerase can ambiguously insert thymine opposite the O⁶-methylguanine in the first round of replication. The thymine on the second round of replication can normally basepair with adenine, converting a G-C basepair into a A-T basepair. Until it is repaired, the O⁶-methylguanine can continue to propagate the mutation. Base excision repair and mismatch repair both recognize the O⁶-methylguanine-thymine basepair.